NOTE TO FILE

Subject: Monsanto's Glyphosate Tolerant Cotton Lines 1445 and 1698

Keywords:

Cotton, *Gossypium hirsutum*, Glyphosate Tolerant, Herbicide Tolerant, CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), Glyphosate oxidoreductase (GOX), Roundup™ Ready, 3'(9)-O-aminoglycoside adenyltransferase (*aad*), APH(3')II.

Background

In a submission dated April 13, 1995, Monsanto provided summary information to support their safety assessment of cotton lines 1445 and 1698. These cotton lines were developed to be tolerant to the herbicide glyphosate.

Molecular Alterations and Characterization of Cotton lines 1445 and 1698

Monsanto reported that they have developed cotton lines 1445 and 1698 by *Agrobacterium* mediated transformation of cotton line Coker 312 with plasmids PV-GHGT07 and PV-GHGT06, respectively. According to information provided by Monsanto, plasmid PV-GHGT06 contains genes for the following enzymes: CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), 3'(9)-O-aminoglycoside adenyltransferase (AAD), and aminoglycoside 3'-phosphotransferase II (APH(3')II). In addition to these genes, Monsanto reported that plasmid PV-GHGT07 contains the gene for the enzyme glyphosate oxidoreductase (GOX).

Monsanto reports that the *cp4-epsps* gene was isolated from *Agrobacterium* sp. strain CP4. Monsanto engineered the *cp4-epsps* gene to contain the chloroplast targeting sequences from the *Arabidopsis thaliana epsps* gene. Monsanto also modified the nucleic acid sequence of the *cp4-epsps* gene to improve expression of the EPSPS protein. This modified gene was expressed in *E. coli* from a PRecA-gene 10L vector. According to Monsanto, comparison of the *in vitro* enzyme activity of the native CP4-EPSPS with CP4-EPSPS enzyme expressed in *E. coli* establishes that the enzyme expressed from the synthetic gene is unaltered. Monsanto contends that their observation that cotton lines 1445 and 1698 are tolerant to the herbicide glyphosate further supports this conclusion.

Monsanto used the kanamycin resistance gene (APH(3')II[1]) as a selectable marker in the initial selection of transformed plant cells during the development of cotton lines 1445 and 1698.

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[1]APH(3')II is an approved food additive permitted in food for human consumption in accordance with § 173.170, and in animal feed in accordance with § 573.130, when used in the development of genetically modified cotton, oilseed rape, and tomatoes.

Based on information provided by Monsanto, the *aad* gene was isolated from the Tn7 transposon and is under the control of its own bacterial promoter. Therefore, the expression of this gene in cotton plants is not expected. Monsanto reported that they confirmed that this gene is not expressed in their cotton lines 1445 and 1698.

According to Monsanto, the *gox* gene is derived from *Achromobacter* sp. strain LBAA. Monsanto engineered the *gox* gene to contain the chloroplast targeting sequences (CTP1).

Based on information provided by Monsanto, both the *epsps* and *gox* genes are each under the control of a promoter from a caulimo virus promoter (CMoVb). The *aph(3')II* gene is under the control of the cauliflower mosaic virus 35S promoter.

Cotton Line 1445

Cotton line 1445 was transformed with plasmid vector PV-GHGT07. Based on restriction fragment analyses of total DNA isolated from transgenic cotton line 1445 and the Coker 312 line, Monsanto has concluded that cotton line 1445 is the result of the insertion of transgenic DNA at one site in the genome of cotton line 1445. According to information provided by Monsanto, the *gox* gene was not integrated into the genome of cotton line 1445. The transgenic DNA is comprised of the following genes: *cp4-epsps*, *aad*, and *aph(3')II*. Based on segregation analysis of progeny, up to the fifth generation, Monsanto concluded that the transgenic DNA is stably inherited in line 1445.

According to Monsanto, cotton line 1445 expresses only two new proteins: 1) CP4-EPSPS; and 2) APH(3')II.

Cotton Line 1698

Cotton line 1698 was transformed with plasmid vector PV-GHGT06. Based on restriction fragment mapping, Monsanto reports that one entire copy of PV-GHGT06 plus 5.6 to 7.4 kb of additional plasmid DNA is integrated at a single site in the genome of cotton line 1698. The additional plasmid DNA includes an intact copy of the *cp4-epsps* gene. Therefore the transgenic DNA in cotton line 1698 is comprised of one copy each of the *aad* and *aph(3')II* genes, and two copies of the *cp4-epsps* gene. Based on their segregation analysis of progeny up to the fifth generation, Monsanto concluded that the transgenic DNA is stably inherited in line 1698.

According to Monsanto, cotton line 1698 expresses two new proteins: 1) CP4-EPSPS; and 2) APH(3')II.

Expressed Proteins

Based on Monsanto's restriction fragment analysis, the only new proteins expected to be expressed in cotton lines 1445 and 1698 are CP4-EPSPS and APH(3')II. Monsanto confirmed this expectation by ELISA assays of extracts from leaf tissue and seeds from cotton lines 1445, 1698, and Coker 312 for CP4-EPSPS, APH(3')II, GOX, and AAD proteins. CP4-EPSPS and APH(3')II proteins were detectable in extracts of leaf tissue and of seeds from lines 1445 and 1698 but not Coker 312. The GOX and AAD proteins were not detected in extracts from leaf tissue, nor was the AAD protein detected in cotton seeds from lines 1445, 1698, and Coker 312.

CP4-EPSPS

Monsanto reported that all crops and microbial food sources contain EPSPS protein. Monsanto noted that there is considerable divergence in the amino acid sequences of EPSPSs which are typically present in foods and that the divergence of the CP4-EPSPS sequence from typical "food" EPSPS sequences is of the same order as the divergence among the "food" EPSPSs. Monsanto concluded that the CP4-EPSPS and other "food" EPSPSs are structurally related as demonstrated by: 1. The amino acid sequence comparison; 2. the homology of active site residues; and 3. the 3-dimensional structure.

Monsanto concluded that CP4-EPSPS does not possess the characteristics of known protein allergens. This conclusion is based on: 1. Comparison of the amino acid sequence of CP4-EPSPS with the amino acid sequence of known food allergens; 2. the observation that although EPSPSs are commonly found in food, none have been reported to be associated with an allergenic response; and 3. the CP4-EPSPS protein is heat labile, and is susceptible to peptic and tryptic digestion, all of which are not common properties of known allergenic proteins.

Monsanto also concluded that the CP4-EPSPS is not toxic. This conclusion is based on: 1. Amino acid sequence comparison of CP4-EPSPS to 1,935 known protein toxins present in the Pir protein, Swissprot, and Genpept protein databases which showed that CP4-EPSPS does not share sequence homology with any toxin sequences in these databases; and 2. an acute gavage study of the CP4-EPSPS protein in the mouse which showed no adverse effects when administered up to 572 mg/kg CP4-EPSPS. They noted that proteins that are known to be toxic to humans act via acute mechanisms.

Compositional Analysis

Based on the nature of the genetic modification, it is expected that cotton lines 1445 and 1698 would not differ materially in composition from other cotton varieties.

To confirm this expectation, Monsanto analyzed the composition of cottonseed, full fat flour, cottonseed meal, and cottonseed oil obtained from lines 1445 and 1698 compared with Coker 312 as a control.

Cottonseed

According to Monsanto, cottonseed is primarily used as cattle feed, with smaller proportions of meal fractions used in feed for poultry, sheep, catfish, and swine.

Compositional (proximate) analyses were reported by Monsanto for cottonseed from cotton lines 1445, 1698, and Coker 312. Components measured were protein, fat, moisture, and ash. Carbohydrate and calories were calculated from these values. Gossypol levels were determined in the seed, full fat flour, and toasted meal.

Monsanto reported no significant differences between cotton lines 1445, 1698, and Coker 312 for: % fat, % ash, calories/100g, and % moisture. Significant differences in the protein and carbohydrate levels between cotton lines 1445 and 1698 compared with Coker 312 were observed at the 5% significance level using a pairwise t-test. Monsanto argues that because the carbohydrate levels are determined by calculation, and not by direct measurement, the differences in carbohydrate level are a result of the increased level of protein and therefore are not considered meaningful. However, Monsanto noted that all values for cotton lines 1445, 1698, and Coker 312 fall within reported ranges, and therefore, the differences observed are not meaningful. In addition, Monsanto reported no significant differences in the amino acid profiles of cotton lines 1445 and 1698, compared to Coker 312.

Because EPSPS catalyzes a step in the aromatic amino acid biosynthetic pathway, Monsanto examined the influence of EPSPS expression on the levels of aromatic amino acids in cottonseed. Monsanto reported no statistically significant increase in aromatic amino acids (tyrosine, phenylalanine, or tryptophan) in cotton lines 1445 and 1698 when compared to Coker 312.

Monsanto reported that gossypol levels detected in cottonseed derived from line 1698 are significantly lower than gossypol levels detected in seed derived from cotton line Coker 312. In contrast, Monsanto reported that the gossypol level observed in seed derived from cotton line 1445 is significantly higher than observed in cottonseed derived from Coker 312. They also reported that even though gossypol levels were highly variable among sites, the observed levels of gossypol in cottonseed derived from lines 1445, 1698, and Coker 312 are all within the reported range for cotton varieties grown under various field conditions. Monsanto concluded that the observed differences in gossypol levels are not meaningful.

Recognizing that aflatoxin produced by Aspergillus fungi can be a contaminant of cottonseed, Monsanto analyzed all three lines for aflatoxin. Monsanto reported that neither the genetically modified lines (1445 and 1698), nor the parental line (Coker 312) contained detectable levels of aflatoxin[2].

Full Fat Flour and Cottonseed meal

Monsanto analyzed the gossypol levels of full fat flour and cottonseed meal derived from cotton lines 1445, 1698, and Coker 312. Toasted meal derived from all three lines was reported to contain bound gossypol, but not free gossypol, however, free gossypol was detected in full fat flour derived from all three lines.

Cottonseed Oil

Monsanto compared the level of α-tocopherol and the fatty acid profile of oil derived from cotton lines 1445, 1698, and Coker 312. Monsanto concluded that levels of α-tocopherol are similar in cottonseed oil derived from all three lines and are within the reported range of α-tocopherol levels in cottonseed oil.

Monsanto observed no significant differences in the lipid content and fatty acid composition of the unrefined cottonseed oil of cotton lines 1445 and 1698 when compared to unrefined cottonseed oil derived from Coker 312.

Monsanto reported that the fatty acid profiles of refined oil from cotton lines 1445, 1698, and Coker 312 are similar and within reported ranges. They noted that the predominant fatty acids detected were: linoleic, oleic, and palmitic as anticipated for refined and unrefined cottonseed oil. The levels of dihydrosterculic, sterculic and malvalic acid in refined and unrefined cottonseed oil were also measured by Monsanto. No significant differences were detected by Monsanto in the levels of these fatty acids in both refined and unrefined cottonseed oil derived from cotton lines 1445 and 1698 when compared with Coker 312.

Wholesomeness Studies

Monsanto conducted a 28 day rat wholesomeness study of ground unprocessed cottonseed, a 10 week catfish wholesomeness study of processed cottonseed meal, and a 5 day quail wholesomeness study of unprocessed cottonseed meal. Based on these studies, Monsanto concluded that unprocessed cottonseed and unprocessed cottonseed meal, derived from both lines 1445 and 1698, are acceptable for use in animal feed.

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[2]The detection limit for aflatoxin is 1 ppb.

Conclusions

Monsanto has concluded that their cotton lines 1445 and 1698 are: ". . . not significantly altered within the meaning of 21 CFR 170.30(f)(2) when compared to Coker 312 the commercial variety of cotton from which it was developed and other cotton varieties presently being commercially grown." At this time, based on Monsanto's description of its data and analyses, the Agency considers Monsanto's consultation on their cotton lines 1445 and 1698 to be complete.

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